Subcellular localization of proteasomes and their regulatory complexes in mammalian cells

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Proteasomes can exist in several different molecular forms in mammalian cells. The core 20S proteasome, containing the proteolytic sites, binds regulatory complexes at the ends of its cylindrical structure. Together with two 19S ATPase regulatory complexes it forms the 26S proteasome, which is involved in ubiquitin-dependent proteolysis. The 20S proteasome can also bind 11S regulatory complexes (REG, PA28) which play a role in antigen processing, as do the three variable γ-interferon-inducible catalytic β-subunits (e.g. LMP7). In the present study, we have investigated the subcellular distribution of the different forms of proteasomes using subunit specific antibodies. Both 20S proteasomes and their 19S regulatory complexes are found in nuclear, cytosolic and microsomal preparations isolated from rat liver. LMP7 was enriched approximately two-fold compared with core α-type proteasome subunits in the microsomal preparations. 20S proteasomes were more abundant than 26S proteasomes, both in liver and cultured cell lines. Interestingly, some significant differences were observed in the distribution of different subunits of the 19S regulatory complexes. S12, and to a lesser extent p45, were found to be relatively enriched in nuclear fractions from rat liver, and immunofluorescent labelling of cultured cells with anti-p45 antibodies showed stronger labelling in the nucleus than in the cytoplasm. The REG was found to be localized predominantly in the cytoplasm. Three- to six-fold increases in the level of REG were observed following γ-interferon treatment of cultured cells but γ-interferon had no obvious effect on its subcellular distribution. These results demonstrate that different regulatory complexes and sub-populations of proteasomes have different distributions within mammalian cells and, therefore, that the distribution is more complex than has been reported for yeast proteasomes.

Key words: ATPase complex, interferon-γ, PA28, 19S complex, 11S regulator.

INTRODUCTION

Proteasomes play a major role in non-lysosomal proteolysis in eukaryotic cells (reviewed in [1,2]). They are responsible for the breakdown of short-lived proteins and play a role in transcriptional regulation and in cell cycle control. Proteasomes are also involved in antigen processing. The structural prototype is the proteasome isolated from *Thermoplasma acidophilum*, which has four stacked heptameric rings forming an $\alpha_7\beta_7\beta_7\alpha_7$ cylindrical structure with the catalytic sites located in the β-subunits facing the inside of the cylinder. Cleavage of an N-terminal propeptide from β-subunits accompanies assembly of the complex and exposes the N-terminal threonine residue, which acts as the catalytic nucleophile. 20S proteasomes in animal cells are made up of at least 14 different subunits. Seven different α-type subunits make up the two outer rings of the cylindrical structure, and seven β-type proteasome subunits make up each of the two inner rings. There are three extra β-type proteasome subunits in animal cells compared with yeast. These three extra subunits, LMP2, LMP7 and MECL1, are inducible by γ-interferon and have been implicated in the antigen processing function of proteasomes (reviewed in [3]). A recent report [4] suggests co-operative incorporation of the inducible subunits into immuno-proteasomes replacing the three other closely related β-type subunits.

A regulatory complex (called 19S regulatory complex, 19S cap, PA700 or ATPase complex) binds to each end of the 20S proteasome to form the 26S proteasome. The regulatory complexes contain approximately 20 different subunits, which vary in size from 25–110 kDa [5,6]. Six of the subunits have ATPase activity. One of the non-ATPase regulatory subunits has been demonstrated to bind multi-ubiquitin chains [7], and the 26S form of the proteasome is responsible for the breakdown of proteins by the ubiquitin–proteasome pathway [8] as well as for some ubiquitin-independent protein degradation.

A second proteasome regulatory complex, the 11S regulator (REG) [9] or PA28 [10], like the 19S regulator, activates the multiple peptidase activities of 20S proteasomes. However, it differs from the 19S complex in two respects. So far, it has not been shown to activate protein degradation by the 20S proteasome, and it does not require ATP for assembling with and activating the 20S proteasome. REG is a ring-shaped complex consisting of two types of subunits, α and β. The two polypeptides have similar molecular masses of approximately 30 kDa. They are products of distinct but homologous genes and their primary structures are approximately 50% identical [11]. The two sub-

Abbreviations used: ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; REG, 11S regulatory complexes.

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units were shown to have significant sequence identity [about 35 %] with a previously described protein, the nuclear Ki antigen [12] or REGγ, which can also activate the proteasome [13]. The function of REG is not well understood although γ-interferon induces synthesis of REG α- and β-subunits and there are results suggesting a role in antigen processing [14].

We have shown previously, by immunogold electron microscopy, immunofluorescence and subcellular fractionation methods using polyclonal antibodies, that 20S proteasomes are localized in the nucleus and in the cytoplasm of mammalian cells [15,16]. A small percentage of the total proteasomes (< 20 %) are loosely associated with the cytoplasmic surface of the endoplasmic reticulum (ER) [15,16]. In contrast, the ER/nuclear envelope appears to be the major site for proteasomes in fission and budding yeast [17,18]. The present studies were undertaken to examine the subcellular distribution of the different subpopulations of proteasomes in mammalian cells using subunit-specific antibodies and also to investigate the effects of γ-interferon on the distribution of proteasomes and their regulatory complexes. The results show differences in the distribution of different subpopulations of 20S and 26S proteasomes, and also in the 11S regulatory complexes. Such differences are probably significant for their functions and may vary in different cell types.

EXPERIMENTAL

Purification of proteasome complexes and REG

Proteasomes (20S and 26S) were purified from rat liver as described previously [19,20]. Chymotrypsin-like activity of proteasomes was assayed [19] using 10–40 μM succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Peptide Institute, Japan) as substrate. Recombinant REGα was purified as described previously [13].

Preparation of subcellular fractions from rat liver and separation of 20S and 26S proteasomes by gel filtration

Subcellular fractionation of rat liver was carried out as described previously [16] and nuclear, cytosolic and microsomal fractions used for immunoblot analysis. For separation of 26S and 20S proteasomes by gel filtration, livers were washed and then homogenized in a Waring blender in 4 vol. of 20 mM Tris/HCl buffer, pH 7.5, containing 20 % (v/v) glycerol, 1 mM 2-mercaptoethanol, 0.1 mM EDTA and 5 mM ATP for 3×30 s at full speed with 1 min intervals. The homogenate was then centrifuged for 1 h at 95000 g and the resulting supernatant separated by gel filtration. Gel filtration was carried out using a Superose 6 FPLC column (Pharmacia) equilibrated with 20 mM Tris/HCl buffer, pH 7.5, containing 100 mM NaCl and 10 % (v/v) glycerol.

Antibodies and immunoblotting

Mouse monoclonal antibodies, MCP72 and MCP257 [21], which recognize the C8 and C9 α-type proteasome subunits, respectively, were used along with rabbit polyclonal anti-20S proteasome antibodies [22] to investigate the total proteasome population. Mouse monoclonal antibodies to the 26S proteasome subunits p45 (p45-110) and TBP1 (TBP1-19) were raised to recombinant proteins. Antibodies to the inducible proteasome β-subunits, LMP2 and LMP7, were also mouse monoclonal antibodies (LMP2-13 and LMP7-1). Rabbit polyclonal antibodies against the 26S proteasome subunit S3 (p91A) were a gift from Dr Amar-Costesec (Universite Catholique de Louvain, Brussels, Belgium) [23]. Anti-S12 antibodies were raised in rabbits as described previously [24] and affinity purified against purified rat liver 26S proteasomes before use. Anti-REG polyclonal antibodies were raised against REG (anti-REG) or against ubiquitin fusion proteins to a unique region in REGα and REGβ for subunit specific antibodies. REGγ antibodies were rabbit polyclonal antibodies raised against a γ-specific peptide [25]. Anti-REG antibodies were affinity purified against recombinant REGα protein before use.

For immunoblot analysis, samples containing known amounts of protein [26] were run on SDS/PAGE gels [27] and immunoblotting was carried out using either an enhanced chemiluminescence (ECL) detection kit (New England Nuclear) or an alkaline phosphatase detection system, as indicated in the Figure and Table legends. Quantification of immunoblots was carried out by densitometric analysis. Purified 20S proteasomes, 26S proteasomes and recombinant REGα were used to establish the linearity of the method and as standards for the quantification.

Cell culture

Human embryo lung L132 cells were grown in Dulbecco's modified Eagle's medium (GIBCO BRL) supplemented with 10 % (v/v) new-born bovine serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) in a humidified atmosphere of 5 % (v/v) CO_2/air.

Immunofluorescence microscopy

Immunofluorescent labelling was carried out as described previously [28], except that cells were fixed in methanol or paraformaldehyde and incubations with primary antibodies were for 2 h at room temperature followed by incubation with pre-immune serum for 15 min and then incubation overnight at 4 °C with rabbit anti-mouse fluorescein-conjugated antibodies. For controls, primary antibodies were omitted. A Leica upright confocal microscope was used to examine the slides. For immunofluorescence studies to investigate the effect of γ-interferon, subconfluent cultures were grown in the presence or absence of 250 units/ml of γ-interferon for 2–3 days.

RESULTS

Proteasome regulatory subunits occur only in complexes in rat liver

Rat liver extracts, which were prepared in the presence of ATP and glycerol to stabilize 26S proteasomes, were fractionated by gel filtration on a Superose 6 column (Figure 1). Assays of rat liver 26S and 20S proteasomes separated on this column showed maximal 26S proteasome activity in fractions 21–23 and maximum 20S proteasome activity in fractions 25–27 (Figure 1A). Assays of activity with succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin showed that most but not all of the activity measured using this substrate was due to proteasomes. An additional small peak of hydrolytic activity of a lower molecular-mass protease was detected in fractions 33–34 of fractionated rat liver extracts (Figure 1A) and in equivalent fractions from cultured cells. The substrate should therefore not be regarded as completely specific for proteasomes, although in thymocyte extracts, where there are relatively higher levels of proteasomes, the assay does appear to be specific [24].

Immunoblots were carried out with the fractions from the gel-filtration column (Figure 1B) to investigate the distribution of individual subunits of proteasomes and their regulators in different complexes. Antibodies against the chosen subunits of

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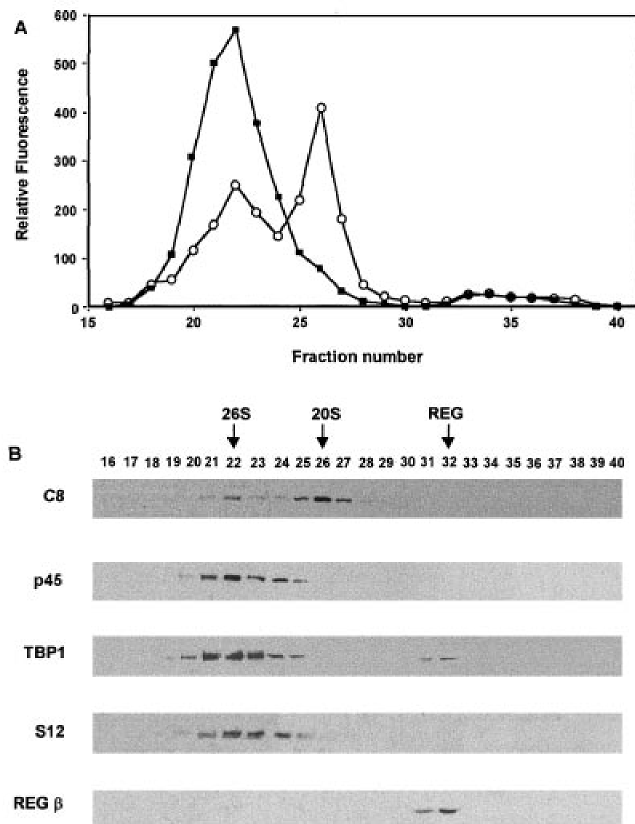

Figure 1 Immunoblot analysis of subunits in proteasome complexes from rat liver

Soluble extracts of rat liver were fractionated by gel filtration on a Superose 6 column as described in the Experimental section. (**A**) Assays for proteasome activity were performed with succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin as substrate under conditions optimized for 26S proteasomes (+ATP, ■) and for activation of latent 20S proteasomes (+0.02% SDS, ○). (**B**) Samples of each fraction (50 μl) from the gel filtration were separated by SDS/PAGE and immunoblotted using anti-C8 antibodies, anti-regulatory complex subunits p45, TBP1 and S12 antibodies and anti-REGβ antibodies, as indicated in the Figure. Free subunits, if present, would be expected to appear in fractions 35–40 (molecular mass approx. 30–100 kDa).

20S proteasomes (C8, C9, LMP7) or their REG or 19S regulatory complexes (p91A, p45, TBP1, S12) were all found to be specific for the appropriate subunit. The 20S proteasome α-type subunits, C8 (Figure 1B) and C9 (results not shown), were found only in fractions where 26S and 20S proteasomes occur (Figure 1B). The relatively stronger C8 bands in fractions 25–27 compared with 21–23 show that the level of 20S proteasomes is higher than that of 26S proteasomes in rat liver extracts. Quantification of the relative levels of C8 or C9 in 20S and 26S proteasome fractions, using purified complexes as standards, indicated that levels of free 20S proteasomes were always greater than those of 26S proteasomes.

Subunits of the 19S regulatory complex (S12 and the ATPase subunits, p45 and TBP1) were all present predominantly in fractions containing the 26S proteasome (Figure 1B), suggesting that there is little, if any, free 19S complex, and no free subunits were detected for any of these subunits. A small amount of TBP1 was observed in fractions 31–32, which is consistent with the occurrence of this subunit in a modulator complex of molecular mass 250 kDa [29]. p91A was also detected only in fractions containing 26S proteasomes.

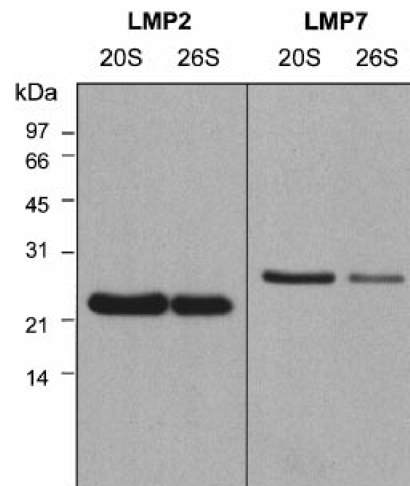

Figure 2 Inducible proteasome subunits, LMP2 and LMP7, are present in purified rat liver 26S proteasome preparations as well as in 20S proteasomes

Purified rat liver 20S proteasomes (1 μg) (left lanes) and purified rat liver 26S proteasomes (3 μg) (right lanes) were separated by SDS/PAGE and the gels were immunoblotted with anti-LMP2 and anti-LMP7 antibodies, as indicated, and detected by ECL.

Under the fractionation conditions employed, reactivity with anti-REG antibodies was seen in fractions 31–32 (Figure 1B) where the free 11S complex would be expected. These results differ from those of Hendil et al. [30] and Ahn et al. [31], who found REG (PA28) associated with 20S proteasomes. The differences in our results can be explained by the limited stability of REG–20S proteasome complexes, which dissociate in the buffer chosen to optimize recovery of 26S proteasomes. The variable γ-interferon-inducible proteasome β-subunits, LMP2 and LMP7, were found to be present in 26S proteasomes as well as in 20S proteasomes. This was confirmed by Western-blot analysis of rat liver 20S and 26S proteasomes, which had been purified to apparent homogeneity (Figure 2). The relative level of LMP7 in the 26S proteasomes was found to be approximately half of that in 20S proteasomes, when quantified by loading equal molar amounts of 20S and 26S proteasomes on to the gels.

Differences in the distribution of proteasome complexes in subcellular fractions from rat liver

Analysis of the localization of 20S proteasomes in subcellular fractions from rat liver has been described previously [16]. The relative distribution of different proteasome regulatory complexes in rat liver subcellular fractions was investigated using the same procedures. In these experiments, polyclonal anti-proteasome antibodies were used to show the distribution of total proteasomes, which was compared with that of immunoproteasomes detected with anti-LMP7 (Table 1). The immunoproteasomes were slightly but significantly enriched in the microsomal fractions and there was a corresponding decrease in the cytoplasmic ratio of LMP7 to total proteasomes (Table 1).

Subunits of the 19S regulatory complexes, like core 20S proteasomes, were found in the nucleus as well as in the cytoplasm and associated with the ER. However, some significant differences were observed in the ratio of different subunits of 19S complexes in different subcellular locations (Table 1), suggesting the possibility that the 26S proteasome can vary in its precise subunit composition. TBP1 and p91A subunits of the 26S proteasome

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Table 1 Distribution of proteasome complexes in subcellular fractions from rat liver

Subcellular fractions were prepared from rat liver. The percentage of each subunit in the three fractions was determined by quantification of immunoblots, where equal amounts of protein had been loaded for each fraction and which had been developed using alkaline phosphatase-conjugated antibodies. The data (means $\pm$ S.D.) were derived from the number of different subcellular fractionation experiments indicated (n). Values obtained with subunit-specific antibodies for immunoproteasome (Immuno 20S) subunit LMP7, for subunits of the 19S regulatory complex (RC), p91A, p45, TBP1, and S12, and for REG were significantly different from those obtained with polyclonal anti-proteasome antibodies at: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0025$ and $^{****}P < 0.0005$ respectively. Statistical analyses were carried out using the Student's t test.

Subunit	Complex	n	Nuclei	Cytosol	Microsomes
Several (anti-proteasome)	20S	4	5.4 ± 1.6	83.2 ± 3.4	11.3 ± 2.0
LMP7	Immuno20S	4	5.5 ± 1.2	$75.0 \pm 3.3^{**}$	$19.5 \pm 2.6^{***}$
p91A	19S RC	4	5.3 ± 0.8	$78.5 \pm 3.1^{*}$	$16.2 \pm 2.9^{*}$
p45 ATPase	19S RC	4	$10.8 \pm 1.5^{***}$	$75.4 \pm 2.6^{**}$	$13.8 \pm 1.5^{*}$
TBP1 ATPase	19S RC	3	6.8 ± 0.7	$77.7 \pm 3.2^{*}$	$15.4 \pm 2.6^{*}$
S12	19S RC	3	$26.1 \pm 11.6^{*}$	$53.3 \pm 7.9^{****}$	$20.6 \pm 4.9^{*}$
REG	11S REG	4	$1.5 \pm 0.4^{***}$	84.6 ± 2.1	13.9 ± 2.1

Table 2 Effect of ATP on ER association of 20S proteasomes and 19S regulatory complexes

The data were derived from immunoblots as described in Table 1. Microsomes were prepared from rat liver homogenized in the absence of ATP (control) or in the presence of ATP (+ATP). Values obtained for each of the subunit-specific antibodies between preparations in the presence and absence of ATP were significantly different at: $^{**}P < 0.01$ and $^{****}P < 0.0005$ respectively. Statistical analyses were carried out using the Student's t test.

Antibody	n	Control	+ATP
Anti-C8	5	12.4 ± 1.3	$21.0 \pm 3.2^{****}$
Anti-C9	6	9.9 ± 2.2	$21.4 \pm 2.6^{****}$
Anti-p45	4	7.2 ± 2.4	$23.4 \pm 7.3^{**}$
Anti-REG	4	12.6 ± 2.0	12.7 ± 2.2

had a similar distribution to each other. The relative amount of 19S regulatory complexes associated with microsomal fractions was significantly greater than 20S proteasomes. There was a significant difference in the distribution of ATPase subunits p45 and TBP1; p45 was significantly higher than TBP1 in nuclear fractions. Even more strikingly, the non-ATPase subunit S12 was relatively low in the cytoplasm but high in nuclear and microsomal preparations. REG showed a similar microsomal/cytosolic ratio to total proteasomes but was only present at very low levels in nuclear preparations (Table 1).

In order to investigate whether addition of ATP, which stabilizes the 26S proteasome, had an effect on the level of proteasomes associated with microsomal fractions, subcellular fractionation was carried out in the presence or absence of ATP. The results shown in Table 2 indicate that both 26S component p45 and core 20S proteasome subunits C8 and C9 were increased in microsomes prepared in the presence of ATP. The level of microsome-associated REG did not change in the presence of ATP.

The level and distribution of 20S and 26S proteasomes in cultured cells

Because proteasome subunits p45 and C8 are found only in proteasome complexes, the ratio of the levels of these subunits can be taken as a measure of the relative amounts of 26S and 20S proteasomes. We have quantified the amounts of these complexes using purified 26S and 20S proteasomes as standards. The immunoblot analysis of extracts for a variety of cultured cells and for rat liver showed that, in all cases, there was an excess of 20S proteasomes. These results are consistent with values for 26S/20S ratios obtained after separation of 26S and 20S proteasomes by gel filtration, carried out under conditions which optimize recovery of intact 26S proteasomes, both with rat liver (Figure 1) and with L132 cells (results not shown). The levels of 20S and 26S proteasomes in L132 cells were determined by quantitative immunoblot analysis using C8 and p45 antibodies with purified 20S and 26S proteasomes as standards. The levels were: 6.9 ± 1.6 μg 20S proteasome/mg total protein (mean $\pm$ S.E., $n = 3$) and 8.5 ± 0.8 μg 26S proteasomes/mg protein (mean $\pm$ S.E., $n = 3$). Thus 20S proteasomes are present in a two- to three-fold molar excess over 26S complexes.

Since p45 subunits were present predominantly in 26S proteasomes, the antibodies were used also to investigate the localization of 26S proteasomes by immunofluorescence microscopy and compared with the distribution of total proteasomes detected using antibody MCP20, which binds to the core 20S proteasome α-type subunit C2. Results from immunofluorescent labelling of cultured human embryo lung L132 cells with MCP20 gave roughly equal intensity of labelling of nucleus and cytoplasm (Figure 3A). Anti-p45 antibodies, on the other hand, showed that p45 is mostly nuclear but is also present in the cytoplasm of L132 cells (Figure 3B). Similar results were obtained with HeLa cells. The results are consistent with those of Peters et al. [32], who found that 19S complexes were present in the nucleus and in the cytoplasm of *Xenopus* oocytes and in mammalian cells.

Localization of the REG in cultured cells

Levels of REGα and β increase 3- to 4-fold following treatment of L-132 cells with γ-interferon (Figure 4). Tanahashi et al. [25] reported an almost complete loss of REGγ protein after interferon treatment in SW620 cells. However, we observed only a minor decrease in its level in L132 cells, as determined by immunoblot analysis and immunofluorescence (Figure 4 and Figure 5). To investigate whether this increase in levels of REG results in any changes in subcellular distribution of the activator, L132 cells were grown in the absence or presence of γ-interferon for 0–4 days. Since the half-life of proteasomes in L-132 cells was about one day and the induction of γ-interferon-inducible proteasome and regulator subunits was maximal within two days, any major effects of γ-interferon on localization of REG would be expected to be observed within a few days. Immunofluorescence studies with anti-REGα and anti-REGβ showed REG, REGα and REGβ to be mostly cytosolic (Figures 5A and 5C). A small amount of labelling was observed in the nucleus but

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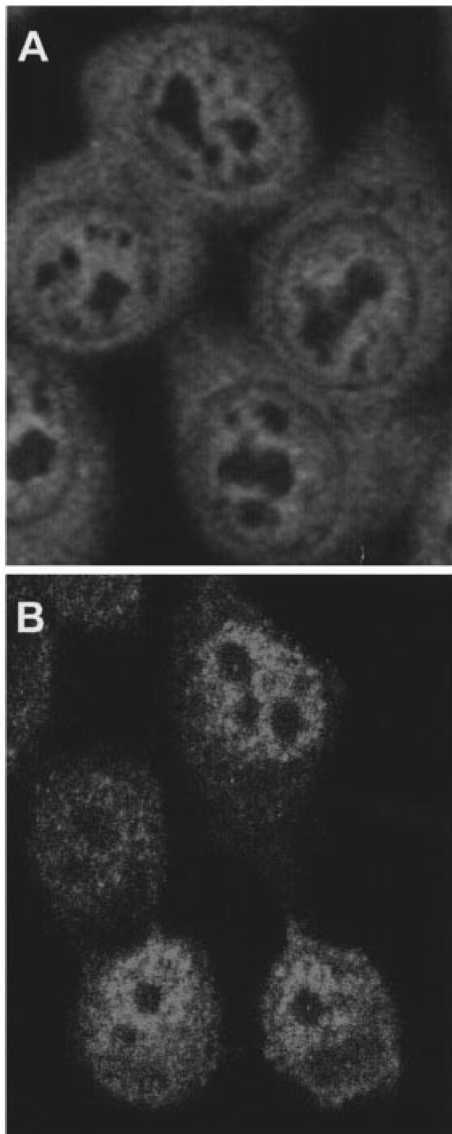

Figure 3 Immunofluorescent labelling of cells for 20S proteasomes and an ATPase subunit of the 19S complex

L132 cells were fixed with 3% paraformaldehyde and immunofluorescence microscopy was carried out as described in the Experimental section with (**A**) anti-C2 antibodies for 20S proteasomes and (**B**) anti-P45 antibodies for 26S proteasomes.

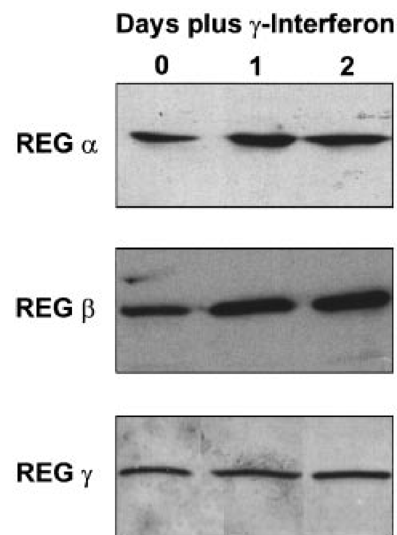

Figure 4 Effect of γ-interferon on levels of REG proteins

Human embryo lung L132 cells were treated with γ-interferon (250 units/ml) for 0, 1 or 2 days, and the total cell extract (100 μg) was separated by SDS/PAGE and the gel was immunoblotted with antibodies against the REG α-subunit, REG β-subunit and the related Ki antigen (REG γ) using subunit-specific antibodies. Relative levels of REG were determined by densitometric analysis.

not in the nucleoli. The presence of γ-interferon had no apparent effect on the distribution of these subunits between the cytoplasm and the nucleus (Figures 5B and 5D). In agreement with its initial description as a nuclear antigen [12], REGγ was found to be present exclusively in the nucleus of L132 cells (Figure 5E) and γ-interferon had no effect on the distribution of REGγ (Figure 5F).

DISCUSSION

The results presented show that subunits of proteasomes and proteasome regulatory complexes do not occur at significant levels as free subunits. The levels of the different complexes can therefore be determined by immunoblot analysis using appropriate subunit-specific antibodies, such as antibodies to C2, C8 or C9 for core 20S proteasomes and antibodies for LMP2 or LMP7 for immunoproteasomes. Since there is little, if any, free 19S complex, anti-p45 antibodies can be used for investigation of the level and distribution of 26S proteasomes as well as for 19S regulatory complexes. A two- to three-fold excess of 20S proteasome was observed both in rat liver and in cultured cell lines. It is possible that the 20S proteasome can function alone, but it is also likely that 11S REG–20S proteasome complexes form *in vivo*. The fact that little if any of this complex was detected in our fractionated cell extracts is probably due to the conditions chosen for the gel filtration, since others have observed these complexes under different conditions [30,31].

The results with antibodies against α-type proteasome subunits (C2, C8, C9) confirm earlier observations that 20S proteasomes are localized in the nucleus and in the cytoplasm, and are associated with the cytoplasmic surface of the ER [15,16]. The microsome-associated proteasomes are localized mainly in the smooth ER and they can be removed from the membranes by extensive washing [16]. The latter may lead to an underestimate of proteasome levels in isolated microsomal fractions. The association of 26S proteasomes with microsomes is highly significant for ER-associated protein degradation, much of which may be ubiquitin dependent (reviewed in [33]). The ratio of 26S proteasomes to 20S proteasomes is greater in the nucleus than in the cytosol, as observed by immunofluorescence microscopy of L132 cells and by subcellular fractionation of rat liver. The fact that the immunofluorescence results are more striking than the immunoblot analysis can be explained by the relatively low volume of liver nuclei [15], and by differences in the methods employed. Peters et al. [32] also found that 19S regulatory complexes of 26S proteasomes were enriched in nuclei in several, but not all, cell types, which they tested by immunofluorescence.

LMP7 was found to be enriched in microsomal proteasomes, as found previously for LMP2 [16], and, since the three γ-

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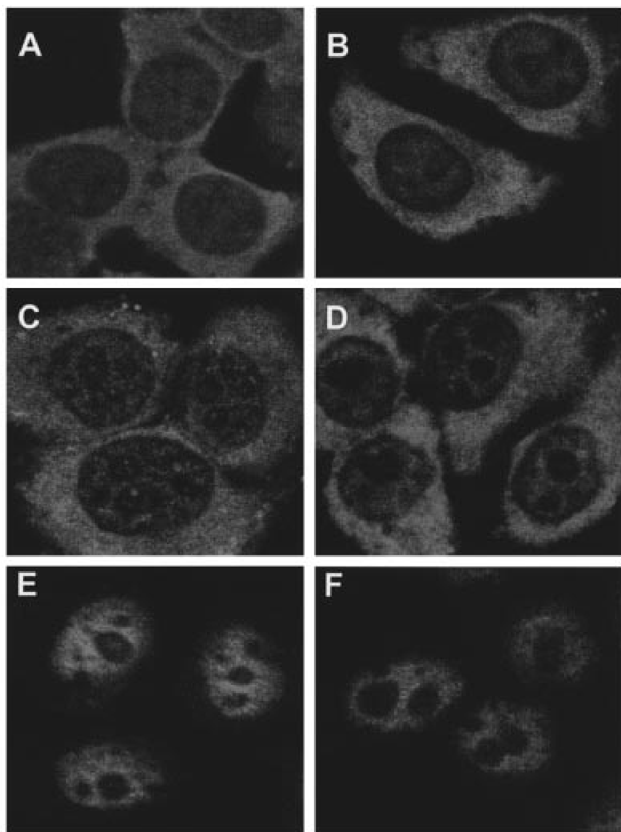

Figure 5 Localization of REG and the effects of γ-interferon

L132 cells were cultured on coverslips in the absence (**A**, **C** and **E**) or presence (**B**, **D** and **F**) of 250 units/ml γ-interferon. Cells were fixed with methanol and immunofluorescence microscopy was carried out using FITC-conjugated secondary antibodies with anti-REG α (**A** and **B**), anti-REG β (**C** and **D**) and anti-REG γ (**E** and **F**) antibodies.

interferon-inducible subunits are apparently incorporated together [4], this result suggests that antigenic peptides may be produced at the ER for efficient transport by the transporter associated with antigen processing (TAP) transporter. The presence of LMP2 and LMP7 in 26S proteasomes (but to a lesser extent than in 20S proteasomes) is consistent with a role for 26S proteasomes and possibly ubiquitin-dependent proteolysis in antigen processing [34].

Some differences in the structure of 19S regulatory complexes have been suggested previously based on results of immunoprecipitation from [^{35}S]methionine-labelled cells [35]. Variations in the distribution of individual subunits of the 19S complexes found in this study provide some evidence in support of this view. Sequence similarities between some non-ATPase components (S12 and POH1 [36]) may also reflect heterogeneity in the regulatory components as well as in the catalytic core of the 26S proteasome.

Our finding that both REGα and REGβ subunits are present mainly in the cytoplasm of liver and L132 cells is in agreement with results of other immunofluorescence studies by Wojcik et al. [37] in NT2 neuronal precursor cells and HeLa S3 cells, although Soza et al. [38] reported that both REG subunits were distributed almost equally between the cytoplasm and nucleus of mouse fibroblast B8 cells. Our results show no major difference in subcellular distribution of REG following treatment with γ-interferon. The distribution of the γ-interferon-inducible REG in the cytoplasm may reflect its proposed role in antigen processing, which is believed to occur predominantly in the cytoplasm. The function of the nuclear REGγ and its role, if any, in interacting with nuclear proteasomes has not been established.

Recent studies have elucidated the localization of proteasomes in yeast [17,18,39]. With green-fluorescent-protein-tagged subunits, proteasomes appeared to be predominantly associated with the nuclear envelope/ER [17,18]. However, using smaller tags, Russell et al. [39] found yeast proteasomes to be distributed throughout the nucleus with only very low levels, if any, in the cytoplasm. Moreover, they found that the level of subunits of the 19S regulatory complex of 26S proteasomes were present in yeast at the same level as a core 20S proteasome subunit. These results are consistent with our observations that 26S proteasomes are relatively more abundant in the nuclei of mammalian cells and that they are distributed throughout the nucleus. Some 26S proteasomes are associated with the ER. The finding that 20S proteasomes are more abundant than 19S regulatory complexes of 26S proteasomes in mammalian cells presumably permits binding of 20S proteasomes to REG complexes which, like the γ-inteferon-inducible proteasome subunits, are absent in yeast. Yeast also appear to lack the proteasome modulator complex [39], which has been observed in mammalian cells [29]. The abundance of proteasomes in nuclei in yeast may reflect their critical role in the regulation of cell-cycle progression in rapidly dividing cells. In contrast, mammalian cells in tissues are generally not rapidly proliferating and proteasomes have some additional specialized functions, such as in antigen processing.

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REFERENCES

1 Coux, O., Tanaka, K. and Goldberg, A. L. (1996) Annu. Rev. Biochem. **65**, 801–847
2 Hilt, W. and Wolf, D. H. (1996) Trends Biochem. Sci. **21**, 96–102
3 Tanaka, K., Tanahashi, N., Tsurumi, C., Yokata, K. Y. and Shimbara, N. (1997) Adv. Immunol. **64**, 1–38
4 Griffin, T. A., Nandi, D., Cruz, M., Fehling, H. J., VanKaer, L., Monaco, J. J. and Colbert, R. A. (1998) J. Exp. Med. **187**, 97–104
5 Dubiel, W. and Rechsteiner, M. (1998) Adv. Mol. Cell Biol. **27**, 129–163
6 Tanaka, K. and Tsurumi, C. (1997) Mol. Biol. Rep. **24**, 3–11
7 Deveraux, Q., Ustrell, V., Pickart, C. and Rechsteiner, M. (1994) J. Biol. Chem. **269**, 7059–7061
8 Ciechanover, A. (1998) EMBO J. **17**, 7151–7160
9 Dubiel, W., Pratt, G., Ferrell, K. and Rechsteiner, M. (1992) J. Biol. Chem. **267**, 22369–22377
10 Chu-Ping, M., Willy, P. J., Slaughter, C. A. and DeMartino, G. N. (1993) J. Biol. Chem. **268**, 22514–22519
11 Ahn, K., Tanahashi, N., Akiyama, K., Hismatsu, H., Noda, C., Tanaka, K., Chung, C., Shimbara, N., Willy, P., Mott, J., Slaughter, C. and DeMartino, G. (1995) FEBS Lett. **366**, 37–42
12 Nikaido, T., Shimada, K., Shibata, M., Hata, M., Sakamoto, M., Takasaki, Y., Sato, C., Takahashi, T. and Nishida, Y. (1990) Clin. Exp. Immunol. **79**, 209–214
13 Realini, C., Jensen, C. C., Zhang, Z., Johnston, S. C., Knowlton, J. R., Hill, C. P. and Rechsteiner, M. (1997) J. Biol. Chem. **272**, 25483–25492
14 Groettrup, M., Soza, A., Eggers, M., Kuehn, L., Dick, T. P., Schild, H., Rammensee, H. G., Kosinowski, U. H. and Kloetzel, P. M. (1996) Nature (London) **381**, 166–168
15 Rivett, A. J., Palmer, A. and Knecht, E. (1992) J. Histochem. Cytochem. **40**, 1165–1172
16 Palmer, A., Rivett, A. J., Thomson, S., Hendil, K. B., Butcher, G. W., Fuertes, G. and Knecht, E. (1996) Biochem. J. **316**, 401–407
17 Enenkel, C., Lehmann, A. and Kloetzel, P. M. (1998) EMBO J. **17**, 6144–6154

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18 Wilkinson, C. R. M., Wallace, M., Morphew, M., Perry, P., Allshire, R., Javerzat, J-P., McIntosh, J. R. and Gordon, C. (1998) EMBO J. **17**, 6465–6476
19 Rivett, A. J., Savory, P. J. and Djaballah, H. (1994) Methods Enzymol. **244**, 331–350
20 Reidlinger, J., Pike, A. M., Savory, P. J., Murray, R. Z. and Rivett, A. J. (1997) J. Biol. Chem. **272**, 24899–24905
21 Kristensen, P., Johnsen, A. H., Uerkvitz, W., Tanaka, K. and Hendil, K. B. (1994) Biochem. Biophys. Res. Commun. **205**, 1785–1789
22 Rivett, A. J. and Sweeney, S. T. (1991) Biochem. J. **278**, 171–177
23 Verlant, V., Amar-Costesec, A., Godelaine, D., Turu, C., Van-Pel, A., De-Plaen, E., Dautry-Varsat, A. and Beaufay, H. (1993) Eur. J. Immunol. **23**, 1727–1730
24 Beyette, J. R., Mason, G. G. F., Murray, R. Z., Cohen, G. M. and Rivett, A. J. (1998) Biochem. J. **332**, 315–320
25 Tanahashi, N., Yokota, K., Ahn, J. Y., Chung, C. H., Fujiwara, T., Takahashi, E., DeMartino, G. N., Slaughter, C. A., Toyonga, T., Yamamura, K., Shimbara, N. and Tanaka, K. (1997) Genes to Cells **2**, 195–211
26 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
27 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
28 Palmer, A., Mason, G. G. F., Paramio, J., Knecht, E. and Rivett, A. J. (1994) Eur. J. Cell Biol. **64**, 163–175
29 DeMartino, G. N., Proske, R. J., Moomaw, C. R., Strong, A. A., Song, X., Hisamatsu, H., Tanaka, K. and Slaughter, C. A. (1996) J. Biol. Chem. **271**, 3112–3118
30 Hendil, K. B., Khan, S. and Tanaka, K. (1998) Biochem. J. **332**, 749–754
31 Ahn, K., Erlander, M., Leturcq, D., Peterson, P., Früh, K. and Yang, Y. (1996) J. Biol. Chem. **271**, 18237–18242
32 Peters, J.-M., Franke, W. W. and Kleinschmidt, J. A. (1994) J. Biol. Chem. **269**, 7709–7718
33 Sommer, T. and Wolf, D. H. (1997) FASEB J. **11**, 1227–1233
34 Rock, K. L. and Goldberg, A. L. (1999) Annu. Rev. Immunol. **17**, 739–779
35 Mason, G. G. F., Murray, R. Z., Pappin, D. and Rivett, A. J. (1998) FEBS Lett. **430**, 269–274
36 Spataro, V., Toda, T, Craig, R., Seeger, M., Dubiel, W., Harris, A. L. and Norbury, C. (1997) J. Biol. Chem. **272**, 30470–30475
37 Wojcik, C., Tanaka, K., Paweletz, N., Naab, U. and Wilk, S. (1998) Eur. J. Cell Biol. **77**, 151–160
38 Soza, A., Knuehl, C., Groettrup, M., Henklein, P., Tanaka, K. and Kloetzel, P. M. (1997) FEBS Lett. **413**, 27–34
39 Russell, S. J., Steger, K. A. and Johnston, S. A. (1999) J. Biol. Chem. **274**, 21943–21952

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